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IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :
VIRGINIE LOUVAIN, ET AL : EXAMINER: TSAY, M.M..
SERIAL NO: 10/518,390 :
FILED: OCTOBER 25, 2005 : ART UNIT: 1656
FOR: THROMBIN CLEAVABLE FACTOR X ANALOGUES:

DECLARATION UNDER 37 C.F.R. § 1.132

COMMISSIONER FOR PATENTS
ALEXANDRIA, VIRGINIA 22313

Sir:

Now comes Bernard Le Bonniec who deposes and states that:

1. I am a graduate of Université Paris 6 and received my PhD degree in
the field of Biochemistry in the year 1986.

2. I have been employed by INSERM, for 13 years in the field of
Thrombosis and Haemostasis.

3. I am a named inventor of the above-identified application.

4. I understand the English language or, at least, that the contents of the Declaration
were made clear to me prior to executing the same.

BLB

5. The following experiments were carried out by me or under my direct supervision and control.

6. The Examples of the present application show that the factor X analogues according to the present invention are cleaved by thrombin and generate amidolytic activity.

In Example 1, beginning on page 9 of the specification, the construction of expression vectors for factor X analogues was disclosed. Specifically, several analogues of factor X were produced, which are as follows (see Table I on page 10 of the specification):

	Factor X analogue	Sequence P₃-P₂-P₁-P'₁-P'₂-P'₃
SEQ ID No 7	GDX-IVG	VPR-IVG
SEQ ID No 8	GDX-IFG	VPR-IFG
SEQ ID No 9	GDX-AVG	VPR-AVG
SEQ ID No 10	GDX-IFR	VPR-IFR
SEQ ID No 11	GDX-SVG	VPR-SVG
SEQ ID No 12	GDX-SFR	VPR-SFR

In Example 4 of the present application (see pages 18-21 of the specification), the rate of cleavage of the factor X analogues by thrombin was evaluated, depending on whether or not this cleavage generates a detectable amidolytic activity. Those experiments also made possible the measurement of the amidolytic activity generated by the activated factor X analogues.

The experiment is a Michaelis Menten kinetics experiment, wherein:

- **K_m** is a constant that is equal to the substrate concentration at which an enzyme reaction proceeds at half the maximum velocity and is associated with the affinity of the enzyme (thrombin) for substrate (the zymogen derived from factor X) ;
- **k_{cat}** gives a direct measure of the catalytic production of product under optimum

conditions ; and

- k_{cat}/K_m represents a measure of enzyme efficiency.

The rate constant was measured, which is directly proportional to the specificity constant (k_{cat}/K_m) of the enzyme (thrombin) for its substrate (the zymogen derived from factor X).

Table V of the present application (see page 21) puts in light the following facts:

- GDX-SVG, GDX-IVG, GDX-IFG and GDX-IFR are cleaved by thrombin but the reaction is too slow for it to be possible to estimate the value of the k_{cat}/K_m ;
- GDX-SFR analogue is cleaved very rapidly but does not generate detectable amidolytic activity ($k_{cat}/K_m=4.10^3 \text{ M}^{-1}.\text{s}^{-1}$) ; and
- GDX-AVG analogue is cleaved by thrombin and has readily detectable amidolytic activity ($k_{cat}/K_m=1.10^2 \text{ M}^{-1}.\text{s}^{-1}$).

This experiment corroborates the fact that VPR-SFR is highly favorable for cleavage by thrombin as described in the previous art.

Moreover, this experiment clearly evidences that VPR-AVG analogue is cleaved by thrombin, in a lesser extent than VPR-SFR, but surprisingly provides a higher amidolytic activity than the others factor X analogues.

7. The Examples of the present application show that the activated form of GDX-AVG analogue interacts with factor Va.

In Example 5 of the present application (see pages 21-38) the activation of prothrombin (which is naturally activated by factor Va and activated factor X).

This experiment clearly illustrates the fact that the addition of factor Va restores the catalytic activity of the activated form of GDX-AVG analogue. In addition, this experiment shows that factor Va does not provide such results with any of the others factor X analogues.

This indisputably indicates that the activated form of GDX-AVG analogue interacts with factor Va, and thus activates prothrombin.

8. The Examples of the present application show that the activated form of GDX-AVG analogue has a higher half life than its native homologue.

In Example 5, the ability of each activated form of the factor X analogues to form a stable covalent complex with antithrombin was determined. For this experiment, the k_{on} of the interaction of antithrombin with the activated forms of the factor X analogue was ascertained.

Physiologically, antithrombin is an inhibitor of the activated form of factor X and the value of its k_{on} for the interaction with activated form of factor X is about $10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$.

In this experiment, a lower value of the k_{on} of a factor X analogue suggests that its interaction with antithrombin is less effective, and thus that said analogue remains active for longer.

The results of this experiment are summarized in Table IX of the present application (see page 36):

- in absence of heparin, the value of k_{on} of the antithrombin for the activated form of GDX-AVG analogue is about $10 \text{ M}^{-1} \cdot \text{s}^{-1}$, i.e. more than 1000 times less than that of its non mutated homologue ($k_{on}=1.2 \cdot 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$), and 10 to 100 times less than the k_{on} values of the others factor X analogues; and
- in presence of heparin, the value of the k_{on} of the antithrombin for the activated form of GDX-AVG ($k_{on}=3.01 \cdot 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) is far lower than for the others factor X analogues.

This observation undoubtedly indicates that, after activation, the GDX-AVG analogue remains active for longer than its non-mutated homologue, which prolongs the procoagulant action of the analogue and therefore considerably reinforces its anti-haemophilic properties.

To confirm this hypothesis, Applicants determined the plasma half life of the activated form of the factor X analogues by measuring their residual activity after incubation for a varying amount of time in a pool of normal human plasmas.

The results are summarized in Table X of the present application (see page 38):

- in presence of heparin, the half life of activated GDX-AVG analogue is about 5 minutes and 30 seconds, whereas the half lives of the others analogues are less than 30 seconds;
- in the absence of heparin, the half life of the activated form of GDX-AVG analogue is notably extended and is about 55 times longer than the others activated factor X analogues.

The foregoing observations would not be apparent to or even expected by the skilled artisan prior to the present application on the basis of his general knowledge or in view of the teachings of Himmelspach et al (US 6,573,071).

9. The Examples of the present application show that the activated form of GDX-AVG analogue has a procoagulant activity.

The procoagulant activity of the activated forms of the factor X analogues was tested. The procoagulant activity of the factor X analogues is compared with that of the normal homologue lacking Gla domain (GD-FX).

Table XI of the present application (see page 40) shows that the activated form of GDX-AVG analogue shortens the clotting time as much as the activated form of the GD-FX analogue, which is not true for the other activated factor X analogues.

This result corroborates the fact that the GDX-AVG analogue clearly has a procoagulant action, unlike the others factor X analogues.

This result is confirmed by Fig. 4 of the present application which compares the procoagulant effect of the GDX-AVG analogue with the GD-FX analogue in factor VIII-depleted (4A) or factor IX-depleted (4B) plasma.

Fig. 4 shows that in the presence of GDX-AVG analogue, the clotting time is shorter than in presence of GD-FX, which undeniably confirms that GDX-AVG analogue is more active than GD-FX analogue.

The fact that GDX-AVG analogue is more active than the GD-FX indicates that an amplification of thrombin generation has indeed taken place in the presence of GDX-AVG.

Example 5 clearly shows that the GDX-AVG analogue leads to a production of at least 26 times more activated forms of factor X.

10. In conclusion, the foregoing clearly shows that:

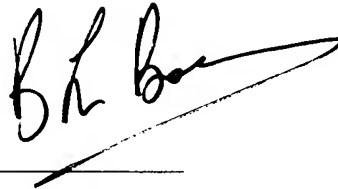
- 1) The GDX-AVG analogue of factor X is efficiently cleaved by thrombin, resulting in the activated form of GDX-AVG analogue;
- 2) the activated form of GDX-AVG analogue provides a high amidolytic activity;
- 3) the activated form of GDX-AVG analogue interacts with factor Va and activates prothrombin;
- 4) the activated form of GDX-AVG analogue has a higher half time than native activated factor X;
- 5) the activated form of GDX-AVG has a procoagulant activity; and
- 6) the activated form of GDX-AVG analogue establishes an autoamplification of thrombin generation.

11. The foregoing evidence clearly establishes that the present invention of an analogue of factor X which has the unexpected result of bypassing the deficient steps of the clotting

cascade. This invention was borne by overcoming the drawbacks of the therapeutic approaches in place prior to the present invention but also establish auto-amplification of thrombin generation in subject suffering from haemophilia. There is no disclosure or suggestion in Himmelsbach et al (US 6,573,071) to select the very specific analogue with a sequence VPR-AVG in the activation peptide, among all factor X analogues disclosed therein. As such, there is nothing expected about the foregoing results when referring to Himmelsbach et al (US 6,573,071).

12. I declare further that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

13. Further Declarant saith not



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Born February 11 1955 in Nancy (France).

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Recent publications related to blood coagulation:

- Chafa O, Tagzirt M, Tapon-Bretonnière J, Reghis A, Fischer AM, Le Bonniec B. Characterization of a homozygous Gly11Val mutation in the Gla domain of coagulation factor X. *Thromb Res.* **2009**;124(1):144-8.
- Smadja DM, Basire A, Amelot A, Conte A, Bièche I, Le Bonniec BF, Aiach M, Gaussem P. Thrombin bound to a fibrin clot confers angiogenic and haemostatic properties on endothelial progenitor cells. *J Cell Mol Med.* **2008** Jun;12(3):975-86.
- Amelot AA, Tagzirt M, Ducouret G, Kuen RL, Le Bonniec BF. Platelet factor 4 (CXCL4) seals blood clots by altering the structure of fibrin. *J Biol Chem.* **2007** Jan 5;282(1):710-20.
- Louvain-Quintard VB, Bianchini EP, Calmel-Tareau C, Tagzirt M, Le Bonniec BF. Thrombin-activatable factor X re-establishes an intrinsic amplification in tenase-deficient plasmas. *J Biol Chem.* **2005** Oct 5;
- Pike RN, Buckle AM, Le Bonniec BF, Church FC. Control of the coagulation system by serpins. *FEBS J.* **2005** Oct;272(19):4842-51.
- Borensztajn K, Chafa O, Le Bonniec B, Wajcman H, Reghis A, Fischer AM, Tapon-Bretonnière J. Inherited factor VII deficiency: identification of two novel mutations (A191V and T239P) in the catalytic domain. *Thromb Res.* **2005**;116(2):115-20.
- Lilla S, Pereira R, Hyslop S, Donato JL, Le Bonniec BF, de Nucci G. Purification and initial characterization of a novel protein with factor Xa activity from *Lonomia obliqua* caterpillar spicules. *J Mass Spectrom.* **2005** Mar;40(3):405-12.
- Saller F, Villoutreix BO, Amelot A, Kaabache T, Le Bonniec BF, Aiach M, Gandrille S, Borgel D. The gamma-carboxyglutamic acid domain of anticoagulant protein S is involved in activated protein C cofactor activity, independently of phospholipid binding. *Blood.* **2005** Jan 1;105(1):122-30.
- Le Bonniec B. [The VKOR target for warfarin identified] *Med Sci (Paris).* **2004** May;20(5):512-4. French.
- Bianchini EP, Pike RN, Le Bonniec BF. The elusive role of the potential factor X cation-binding exosite-1 in substrate and inhibitor interactions. *J Biol Chem.* **2004** Jan 30;279(5):3671-9.
- Chabut D, Fischer AM, Collic-Jouault S, Laurendeau I, Matou S, Le Bonniec B, Helley Low molecular weight fucoidan and heparin enhance the basic fibroblast growth factor-induced tube formation of endothelial cells through heparan sulfate-dependent alpha6 overexpression. *Mol Pharmacol.* **2003** Sep;64(3):696-702.
- Ludeman JP, Pike RN, Bromfield KM, Duggan PJ, Cianci J, Le Bonniec B, Whisstock JC, Bottomley SP. Determination of the P1', P2' and P3' subsite-specificity of factor Xa. *Int J Biochem Cell Biol.* **2003** Feb;35(2):221-5.
- Braud S, Le Bonniec BF, Bon C, Wisner A. The stratagem utilized by the plasminogen activator from the snake *Trimeresurus stejnegeri* to escape serpins. *Biochemistry.* **2002** Jul 2;41(26):8478-84.

Bianchini EP, Louvain VB, Marque PE, Juliano MA, Juliano L, Le Bonniec BF. Mapping of the catalytic groove preferences of factor Xa reveals an inadequate selectivity for its macromolecule substrates.

J Biol Chem. **2002** Jun 7;277(23):20527-34.

Quinsey NS, Whisstock JC, Le Bonniec B, Louvain V, Bottomley SP, Pike RN. Molecular determinants of the mechanism underlying acceleration of the interaction between antithrombin and factor Xa by heparin pentasaccharide.

J Biol Chem. **2002** May 3;277(18):15971-8.

Gaussem P, Dubar M, le Bonniec B, Richard-Lordereau I, Jochemsen R, Aiach M. Dose-effect relationship for several coagulation markers during administration of the direct thrombin inhibitor S 18326 in healthy subjects.

Br J Clin Pharmacol. **2002** Feb;53(2):147-54.

Borgel D, Gaussem P, Garbay C, Bachelot-Loza C, Kaabache T, Liu WQ, Brohard-Bohn B, Le Bonniec B, Aiach M, Gandrille S. Implication of protein S thrombin-sensitive region with membrane binding via conformational changes in the gamma-carboxyglutamic acid-rich domain.

Biochem J. **2001** Dec 1;360(Pt 2):499-506.

Myles T, Le Bonniec BF, Betz A, Stone SR. Electrostatic steering and ionic tethering in the formation of thrombin-hirudin complexes: the role of the thrombin anion-binding exosite-I.

Biochemistry. **2001** Apr 24;40(16):4972-9.

Ludeman JP, Whisstock JC, Hopkins PC, Le Bonniec BF, Bottomley SP. Structure of a serpin-enzyme complex probed by cysteine substitutions and fluorescence spectroscopy.

Biophys J. **2001** Jan;80(1):491-7.

Myles T, Le Bonniec BF, Stone SR. The dual role of thrombin's anion-binding exosite-I in the recognition and cleavage of the protease-activated receptor 1.

Eur J Biochem. **2001** Jan;268(1):70-7.